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Feather keratin as a ligand in an affinity chromatographic technique for isolation of protease from *Trichophyton verrucosum*

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ABSTRACT

A technique of affinity chromatography was developed and optimized for proteases from the post-culture fluid of *Trichophyton verrucosum*. The technique employs porous glass with adsorbed feather keratin or keratin covalently bound to the glass. Modifications of the amounts of proteases introduced into the columns and the manner of elution (pH gradient, buffer concentration, EDTA) made it possible to achieve yields of the isolated enzymes of the order of 80%. The degree of purification of four fractions isolated by the proposed technique was about 6-fold and allowed electrophoretically almost homogeneous enzymatic forms to be isolated. Substrate and inhibition tests on the four chromatographically purified proteolytic enzymes indicated a specifically keratinolytic nature of the enzymes studied.

INTRODUCTION

Chicken feather keratin (K) is a troublesome industrial waste, and utilization of this material would be beneficial. K is particularly difficult to dissolve in any solvent. However, it has been found that part of it can be solubilized in dimethyl sulphoxide (DMSO). The properties of the DMSO-soluble fraction of K have been well characterized elsewhere [1]. K solubilized in DMSO can be precipitated with acetone and then forms a suspension in aqueous buffers. This soluble feather keratin protein (KS) in the form of a suspension was used in our earlier research on the characterization of proteases from various fungal strains [2–4].

The solubilized, suspended K can be used as an adhesive coating, *e.g.*, on silica gel, controlled-pore glass (CPG) or other supports, and activates their surface with the free NH_2 or COOH groups of this protein [6–8]. Such activated supports allowed the use of K-coated matrices either for enzyme immobilization or as affinity sorbents for ligand binding [7].

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In this paper, we describe the use of K-coated supports or KS covalently bound to supports as a ligand for the affinity chromatography of proteases with keratinase-like properties. These matrices have been found to be good materials for the affinity chromatographic separation of keratinase from other proteases present in a medium after *Trichophyton verrucosum* culture.

EXPERIMENTAL

Protease

Trichophyton verrucosum strain V 84/10, obtained from the Central Veterinary Laboratory (Weybridge, U.K.), was grown on a liquid Sabourauds medium [3] in stationary cultures for 4-day periods. After that time, the medium was separated from the culture by centrifugation at 5000 *g* for 15 min. The medium containing the proteases was salted out by 80% ammonium sulphate saturation. The precipitate was dissolved in 0.01 *M* phosphate buffer and dialysed against the same buffer at 4°C. The protease preparation was then desalted on a Sephadex G-25 column (20 × 5 cm I.D.) and lyophilized. This preparation was used for the subsequent affinity chromatographic experiments.

Matrices and column preparation

K protein was solubilized in DMSO as described elsewhere [2]. KS in DMSO contained *ca.* 2% of protein, as determined by the method of Schacterle and Pollack [9]. A 50-ml sample of this solution, containing *ca.* 1 g of protein, was mixed with acetone at a ratio of 1 volume of KS per 3 volumes of cooled acetone (4°C). The protein precipitate which formed was centrifuged at 5000 *g* for 15 min at 4°C and dissolved in 50 ml of 0.01 *M* phosphate buffer (pH 7). This suspension contained about 2% of KS protein and was used for coating the CPG or for covalent binding to CPG. The CPG used in our experiments was in the form of glass beads, produced by Cormay (Lublin, Poland); they had a porosity of about 500 Å and measured 0.2 mm in diameter. The KS suspension described above was mixed with CPG at a ratio of 10 ml/g. Water was completely evaporated at 50°C and KS was adsorbed on the surface of the CPG, as was checked previously [6].

KS covalently bound to CPG was obtained by using alkylaminated CPG (500 Å, 0.2 mm in diameter; Cormay). This CPG was further activated by glutaraldehyde, and then KS was immobilized by the method of Lappi *et al.* [10]. According to this method, KS binding with CPG employed 1 g of the porous glass per 40 mg of KS protein.

Two kinds of KS-coated supports, with KS either adsorbed or covalently bound, were used for affinity chromatographic experiments in columns of 15 × 1.0 cm I.D. The columns were equilibrated with 0.1 *M* phosphate buffer (pH 7.0) containing 5 m*M* zinc chloride.

Affinity chromatography

The protease preparations were applied to the columns, which columns were eluted with the equilibrating buffer to remove unbound proteins (flow-rate 5 ml/min, temperature 4°C). The proteases, bound to the columns, were then desorbed with three types of solutions: (1) 0.1 *M* phosphate–citrate buffer (pH 4.0) containing 5 m*M*

zinc chloride; (2) 0.1 *M* phosphate buffer (pH 7.0) containing 3 m*M* EDTA and 5 m*M* zinc chloride; (3) 0.5 *M* phosphate buffer (pH 7.0) containing 5 m*M* zinc chloride.

Fractions of 10 ml were collected in a fraction collector and their absorbance at 280 nm and protease activity against two substrates were determined. Hydrochloric acid denatured haemoglobin (Hb-HCl) or KS in 2% solutions were used as substrates.

Proteolytic activity

Against Hb-HCl. Denatured Hb-HCl, prepared by the Mycek method [11], was dissolved in 0.1 *M* phosphate buffer (pH 7.0), containing 0.01 *M* zinc chloride to give a 2% solution. A 3-ml volume of this solution was mixed with 0.5 ml of enzyme solution and the mixture was incubated for 1 h at 37°C. The proteolytic reaction was stopped by adding 4.5 ml of 5% trichloroacetic acid (TCA). After 10 min, the mixture was filtered and the filtrate was assayed by the ninhydrin reaction. One proteolytic unit was defined as the amount of enzyme which released 1 μmol/ml of glycine from the Hb-HCl. The amounts of amino acids cleaved by the enzymes from the column ligand (KS), both adsorbed on the support and covalently bound, were found to be insignificant. Owing to the high flow-rate (one chromatographic cycle lasting 80 min), the ligand was digested by the enzymes weakly enough for the absorbance values of the TCA supernatant to be insignificant in relation to the activity.

Against KS. A 1-ml volume of a 2% suspension of KS in 0.1 *M* phosphate buffer (pH 7.0) containing 0.01 *M* zinc chloride was mixed with 1 ml of enzyme solution and incubated for 1 h at 37°C. The proteolytic reaction was stopped by adding 2 ml of 5% TCA solution. After 10 min, the mixture was filtered and the absorbance at 280 nm was determined. One unit of proteolytic activity was defined as the amount of enzyme that caused a 0.01 increase in absorbance at 280 nm per millilitre.

Specific substrate activity of proteases. Proteolytic activities were determined against various proteins (KS, Hb-HCl, casein, azocasein, cytochrome *c*, beef albumin, myoglobin) and expressed in the same units as above. The determinations were performed as follows. Volumes of 3 ml of 2% solutions of the above proteins in 0.1 *M* phosphate buffer (pH 7.0) containing 0.01 *M* zinc chloride were mixed with 0.5 ml of the enzyme solution and incubated for 1 h at 37°C, then 4.5 ml of 5% TCA were added to stop the proteolytic reaction. After 10 min, the mixtures were filtered and the increases in absorbance at 280 nm were measured to determine proteolytic activities. One unit of enzymatic activity was defined as a 0.01 absorbance increase at 280 nm per millilitre.

Changes in proteolytic activity after introduction of inhibitors

The specific effects of inhibitors on crude and purified fractions of proteolytic preparations were determined in the presence of phenylmethane sulphone fluoride (PMSF), soybean trypsin inhibitor (STI), iodoacetamide and *p*-chloromercuribenzoate (pCMB) at 2 m*M* concentrations, 5 mM diazoacetyl-D,L-norleucine methyl ester (DAN), 1 m*M* pepstatin and 10 m*M* EDTA.

Equal volumes of the inhibitors and enzyme solutions were mixed and kept at 37°C for 30 min, then 0.5 ml of the solution was used for proteolytic activity determinations against Hb-HCl or KS as described above. Enzyme solutions kept at 37°C for

30 min with the buffer were used as controls. Activity units were expressed as absorbance increases at 280 nm.

Influence of pH on proteolytic activity

The following buffers were employed: 0.1 *M* glycine–NaCl–HCl (pH 1.2–3.0), 0.1 *M* phosphate–citrate buffer (pH 3.0–7.4) and 0.1 *M* Tris–HCl buffer (pH 7.4–9.0).

Proteolytic substrates, such as Hb-HCl and KS, were prepared in the above buffers and added in 3-ml volumes to 0.5 ml of the enzyme. The mixtures were incubated and proteolytic activities were then determined as described above.

Electrophoretic separation of proteinases

The method of North and Harwood [12] was used with separating gels containing 12% (w/v) acrylamide and 0.2% (w/v) denatured haemoglobin. Buffers and gels were prepared according to Laemmli [13], without the addition of sodium dodecyl sulphate. Samples, concentrated by lyophilization after desalting and containing up to 300 μg of protein, were applied to stacking gels. Electrophoresis was carried out at 4°C and 1 mA per gel from the cathode to the anode until the tracking dye (bromophenol blue) entered the separating gel, and then at 2 mA per gel. After electrophoresis, the gels were incubated for 18 h at 37°C in 0.1 *M* sodium acetate–acetic acid buffer (pH 5.0), then stained with 0.015% nigrosine in 0.1 *M* Tris–HCl buffer (pH 7.5) for 24 h and destained for 2–3 days in the same buffer without nigrosine. Haemoglobin was stained dark. Clear bands showed the presence of proteinase. Densitometric scans of the gels at 540 nm were made, using a TLD 100 densitometer (Vitatron, Dieren, The Netherlands). Denatured haemoglobin for these experiments was obtained from Gurr (Poole, U.K.). Acrylamide was supplied by Serva (Heidelberg, F.R.G.). Nigrosine was purchased from Chemapol (Prague, Czechoslovakia).

RESULTS AND DISCUSSION

Comparative studies were carried out for the optimization of the isolation and purification of the extracellular proteases synthesized by *Trychophyton verrucosum* by affinity chromatography. Two kinds of column packings were applied; one contained CPG coated with the hen KS fraction and the other was packed with alkylamino-CPG with covalently bound hen KS. In both instances KS was employed as a ligand for the proteases from *T. verrucosum*. On the basis of the results presented in Figs. 1 and 2 and in Tables I and II, it can be concluded that the assumptions of the affinity technique were confirmed in practice. The amounts of enzymes and the procedures for their elution (Figs. 1–3, Tables I–III) have been optimized. In the first stage of our work (Figs. 1 and 2 and Tables I and II), a one-step pH change, from 7.0 to 4.0, was applied for protease elution. The column containing K bound to CPG separated the proteases into three fractions. The column in which K was covalently bound to the carrier gave a higher resolution, giving four fractions (Fig. 2). Using columns of the same size (15 × 1.0 cm I.D.) with adsorbed KS and covalently bound KS as ligands, an optimization of the amounts of introduced proteases and the general elution of proteases, and also as the determination of the degree of purification of the fractioned proteins, were carried out (Tables I and II). The overall yields of proteases eluted from the column containing covalently bound KS (Table II) were higher then those

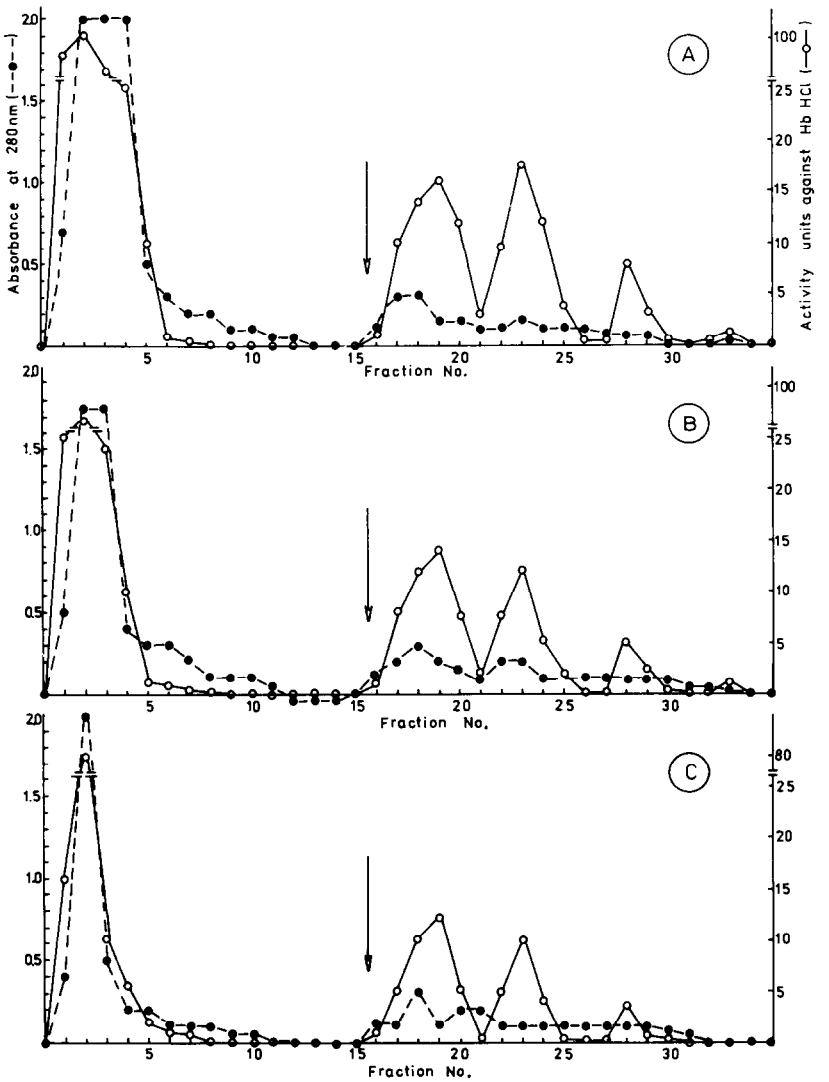

Fig. 1. Elution profiles of proteases (*T. verrucosum*) from columns (15 × 1.0 cm I.D.) packed with CPG with adsorbed KS. The columns contained (A) 15 ml (3770 U), (B) 10 ml (2480 U) and (C) 5 ml (1230 U). The proteases were eluted with a one-step pH change from 7.0 to 4. The buffer changes are marked with arrows; 10-ml fractions were collected.

from the column containing adsorbed KS (Table I). On the other hand, the degree of purification of fractions I–III in Table I and I–III in Table II did not differ significantly. Both columns showed good repeatability and did not lose their properties after multiple use (30 regenerations). This indicates the possibility of applying them on a biotechnological scale in the future.

In further experiments we tried to optimize the elution of proteases from the column with covalently bound KS (Fig. 3, Table III). KS was now applied also as a

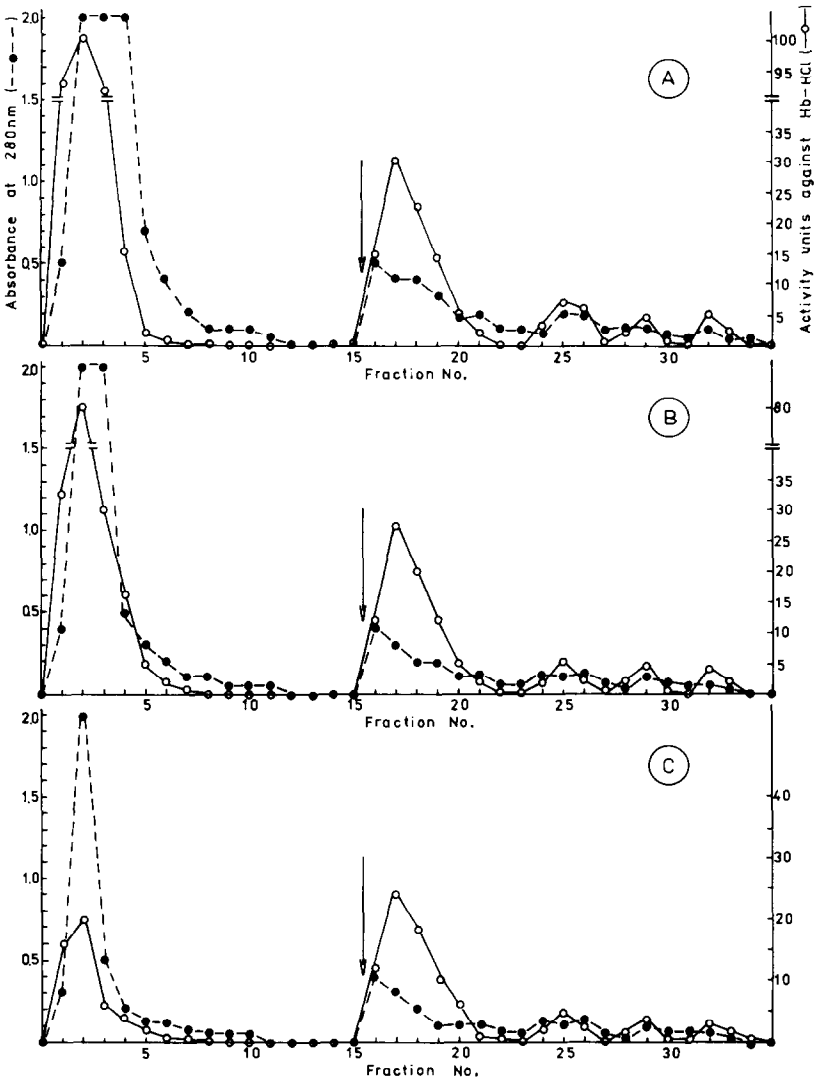

Fig. 2. Elution profiles of proteases (*T. verrucosum*) from columns (15 × 1.0 cm I.D.) packed with CPG with covalently bound KS. The columns contained (A) 15 ml (3510 U), (B) 10 ml (2510 U) and (C) 5 ml (1250 U). The proteases were eluted with a one-step pH change from 7.0 to 4.0. The buffer changes are marked with arrows; 10-ml fractions were collected.

substrate for the determination of proteolytic activity in addition to Hb-HCl, as in the results shown in Figs. 1 and 2. The results shown in Fig. 3 and Table III illustrate three procedures for protease elution from the column containing covalently bound KS: (a) a one-step change of elution pH from 7.0 to 4.0; (b) a one-step change of buffer concentration from 0.1 to 0.5 *M*; and (c) the addition of 3 m*M* of EDTA to the phosphate buffer (pH 7). The overall protease elution from the column, expressed as a percentage, was high (about 85%) and did not change significantly in the different

TABLE I

OPTIMIZATION OF AFFINITY CHROMATOGRAPHY OF PROTEASES FROM *T. VERRUCOSUM* ON THE CPG COLUMN (15 × 1.0 cm I.D.) WITH KS ADHESIVELY BOUND TO THE SUPPORT

Yield and purification factors of proteases. The activity was determined against Hb-HCl as the substrate.

Units applied to the column (μmol Gly/ml)	Units eluted from the column (μmol Gly/ml)	Yield (%)	Fraction I		Fraction II		Fraction III	
			Purification efficiency	Yield (%)	Purification efficiency	Yield (%)	Purification efficiency	Yield (%)
3770	1070	28.4	2.8	11.1	3.1	10.3	1.7	2.7
2480	780	30.5	3.0	14.5	3.1	10.9	1.7	3.2
1230	550	44.7	3.0	21.9	3.1	14.6	1.6	3.2

TABLE II

OPTIMIZATION OF AFFINITY CHROMATOGRAPHY OF PROTEASES FROM *T. VERRUCOSUM* ON THE CPG COLUMN (15 × 1.0 cm I.D.) WITH KS COVALENTLY BOUND TO THE SUPPORT

The protease elution was performed by changing the pH from 7.0 to 4.0. Yield and purification factors of proteases. The proteolytic activity was determined against Hb-HCl as substrate.

Units applied to the column (μmol Gly/ml)	Units eluted from the column (μmol Gly/ml)	Yield (%)	Fraction I		Fraction II		Fraction III		Fraction IV	
			Purification efficiency	Yield (%)	Purification efficiency	Yield (%)	Purification efficiency	Yield (%)	Purification efficiency	Yield (%)
3510	1060	30.2	3.1	23.9	1.8	4.3	1.2	1.7	1.9	1.7
2510	880	35.1	2.9	28.7	1.8	3.6	1.3	2.4	1.6	1.6
1250	790	63.2	2.9	54.4	1.6	4.8	1.3	3.2	1.1	1.6

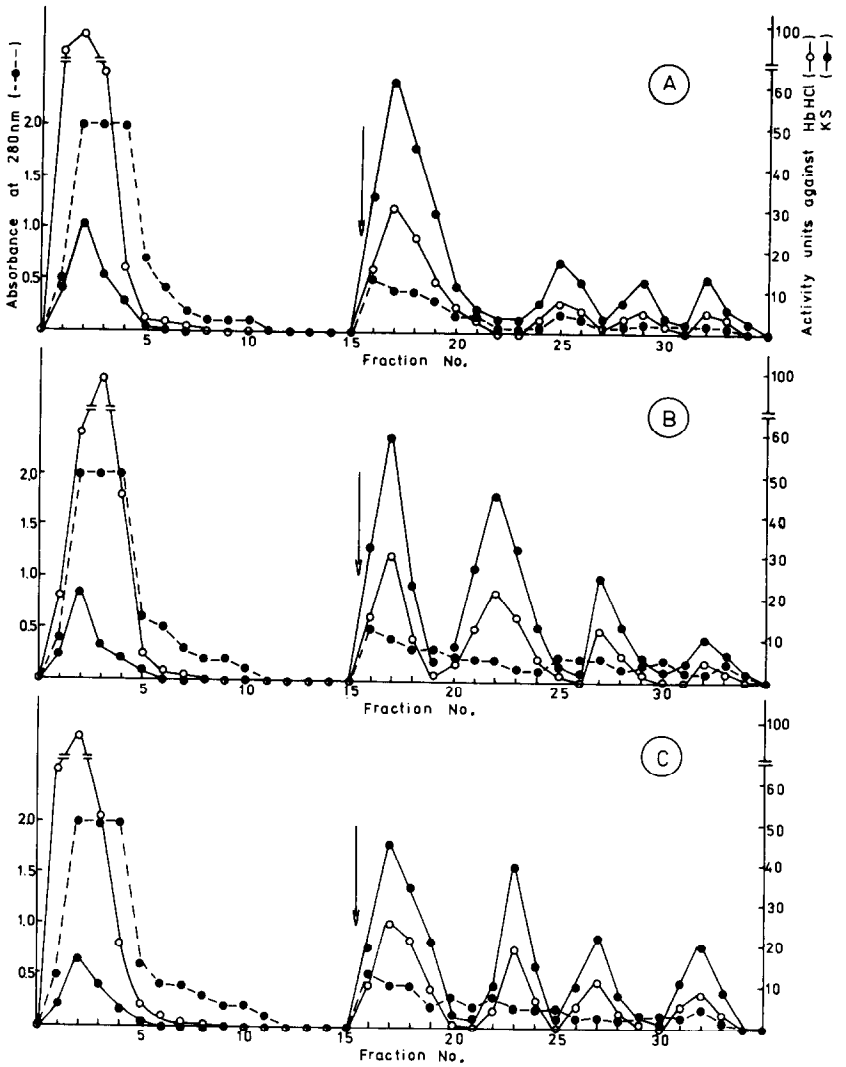

Fig. 3. Elution profiles of proteases (*T. verrucosum*) from columns (15 × 1.0 cm I.D.) packed with CPG with covalently bound KS. The proteases were eluted (A) by a pH change from 7.0 to 4.0; (B) by a concentration change of phosphate ions (pH 7.0) from 0.1 to 0.5 *M*; (C) by application of 3 m*M* EDTA in 0.1 *M* phosphate buffer (pH 7.0); 10-ml fractions were collected.

procedures (Table III). However, the degree of purification of particular protease fractions and their elution yield seemed best when buffer of pH 4.0 was applied to elute them from the column (Table III).

Substrate specificity of the four isolated proteolytic fractions from *T. verrucosum* was also determined, using seven animal proteins. In these determinations the same proteolytic activity was used. Assuming that *T. verrucosum* contains proteases with keratinolytic properties, the enzymatic activity determined against KS as a sub-

TABLE III

OPTIMIZATION OF AFFINITY CHROMATOGRAPHY OF PROTEASES FROM *T. VERRUCOSUM* ON THE CPG COLUMN (15 × 1.0 cm I.D.) WITH KS COVALENTLY BOUND TO THE SUPPORT

The protease elution was performed either by changing the pH from 7.0 to pH 4.0 or phosphate ion concentration from 0.1 to 0.5 *M* or with the use of 3 m*M* EDTA solution. The proteolytic activities were determined using soluble KS protein as a substrate.

Method of elution	Units applied to the column (μmol Gly/ml)	Units eluted from the column (μmol Gly/ml)	Yield (%)	Fraction I		Fraction II		Fraction III		Fraction IV	
				Purification efficiency	Yield (%)	Purification efficiency	Yield (%)	Purification efficiency	Yield (%)	Purification efficiency	Yield (%)
pH change	3000	2540	84.0	7.1	56.0	5.1	12.0	4.3	6.0	6.9	6.0
Ion concentration change	3559	3040	85.4	3.6	32.0	5.1	29.5	3.3	11.0	2.0	5.1
EDTA elution	3050	2690	88.2	5.0	39.3	5.6	21.6	4.9	12.8	7.5	11.8

TABLE IV

SUBSTRATE SPECIFICITY OF EXTRACELLULAR PROTEASES FROM *T. VERRUCOSUM* AGAINST SEVERAL ANIMAL PROTEINS

The fractions of the proteases from *T. verrucosum* were obtained by affinity chromatography on the CPG column with covalently bound KS. Proteolytic activities were determined in the same units (absorbance changes at 280 nm) and calculated as percentage values, using the enzymatic activity against KS as a substrate as 100%.

Substrate	Activity (% of control)			
	Fraction I	Fraction II	Fraction III	Fraction IV
KS	100	100	100	100
Hb-HCl	49	57	67	75
Casein	29	29	27	22
Azocasein	37	40	40	38
Cytochrome *c*	54	114	57	27
Albumin	15	23	47	42
Myoglobin	24	34	40	35

TABLE V

INFLUENCE OF INHIBITORS ON CHANGES IN PROTEOLYTIC ACTIVITY OF EXTRACELLULAR PROTEASES FROM *T. VERRUCOSUM* (FRACTIONS I, II, III AND IV)

The fractions were separated by affinity chromatography on the CPG column with covalently bound KS. The examination of the effect of the inhibitors was carried out using Hb-HCl or KS as substrates.

Inhibitor	Substrate	Activity remaining (% of control)			
		Fraction I	Fraction II	Fraction III	Fraction IV
None (control)	KS	100	100	100	100
	Hb-HCl	100	100	100	100
Pepstatin	KS	90	100	90	40
	Hb-HCl	30	44	30	40
DAN	KS	100	100	100	75
	Hb-HCl	90	100	90	80
pCMB	KS	98	99	98	95
	Hb-HCl	95	97	95	90
Iodoacetamide	KS	100	100	100	95
	Hb-HCl	96	100	98	95
EDTA	KS	14	32	46	30
	Hb-HCl	25	36	60	55
o-Phenanroline	KS	15	5	10	20
	Hb-HCl	20	10	20	50
PMSF	KS	42	33	38	25
	Hb-HCl	61	44	53	45
STI	KS	40	42	50	35
	Hb-HCl	48	45	53	40

strate was assumed to be 100%, and it was compared with the proteolytic activities of the six other substrates (Table IV). The results in Table IV show the validity of these assumptions. The proteolytic activities of all four enzymatic fractions were highest against KS. An exception was the activity of fraction II, determined against cytochrome *c* (Table IV), but the general conclusion implied by Table IV seems to be clear: the proteolytic activities of the four fractions of proteases from *T. verrucosum* examined reveal a high substrate specificity against keratin protein.

Further characterization of the chromatographically isolated fractions of proteases from *T. verrucosum* is presented in Table V, which shows the results of compar-

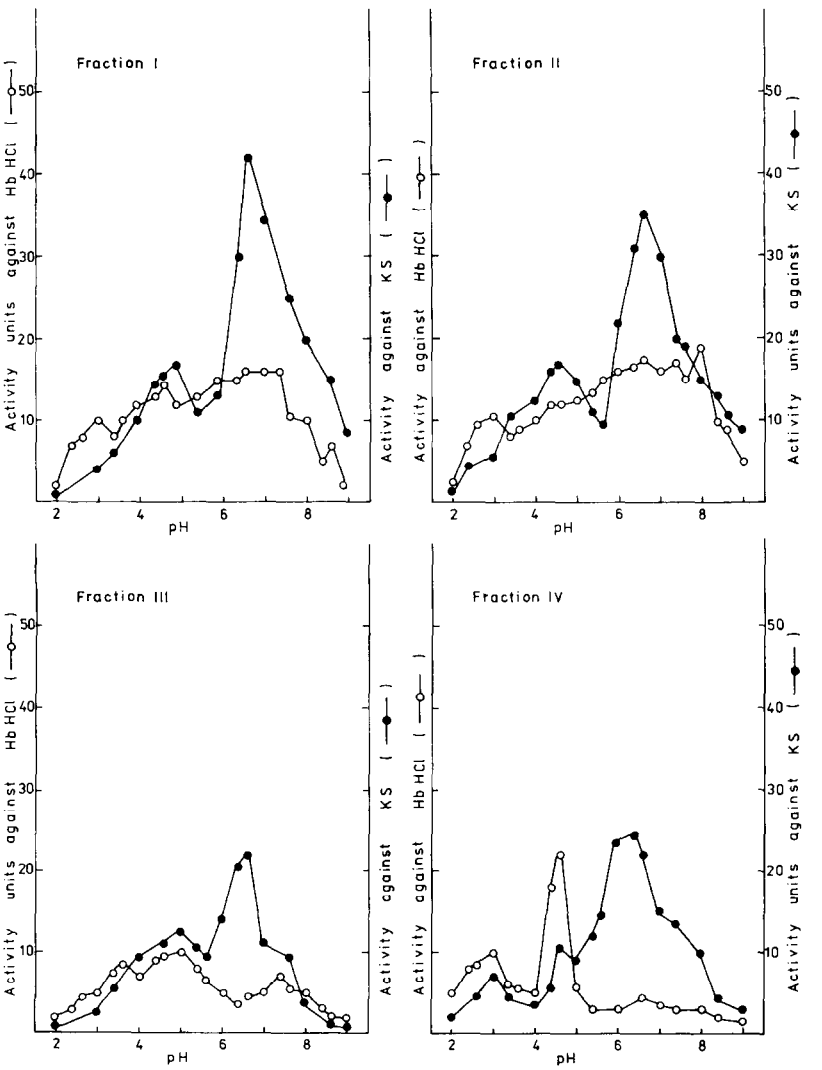

Fig. 4. Effect of pH on proteolytic activity of extracellular proteases of *T. verrucosum*. Fractions I–IV were obtained by affinity chromatography on the CPG with covalently bound KS. Protease activities were determined in the presence of Hb-HCl or KS as substrates.

ative studies of the effect of eight inhibitors of proteases on the proteolytic activities of four forms of enzymes. In these experiments, two substrates (Hb-HCl and KS) were used in parallel. The metal dependence of all four fractions is indicated by the strong effects of such inhibitors as EDTA and *o*-phenanthroline on the proteolytic activities (Table V). It is assumed that the active centre of the four protease fractions examined also contains serine, in view of the strong inhibition of these enzymes by PMSF and STI (Table V).

Fig. 4 presents the results of determinations of the optimum pH for the four purified proteolytic fractions from *T. verrucosum* against Hb-HCl or KS. Against KS, fractions I–III evidently possessed two optimum pH values, the main one at pH 6–7 and a lesser one at pH 4–5. Under the conditions applied, fraction IV had only one optimum at pH 6–7. On the other hand, the activity of this fraction in the presence of Hb-HCl had its optimum at pH 4–5 (Fig. 4). The activities of the other fractions (I–III), determined against Hb-HCl, did not show any evident pH optima (Fig. 4).

Electrophoretic studies, illustrated in Fig. 5, concerned the crude enzymatic preparation and the chromatographically isolated fraction with proteolytic activity from *T. verrucosum*. The results show a high degree of purification of the examined forms of enzymes from the proteins, which did not have, under our chromatographic conditions, any affinity to the active supports used. In this respect, a close similarity of the chromatographically separated fractions was achieved, especially of fractions II, III and IV. The results of these experiments suggest that the isolated fractions are

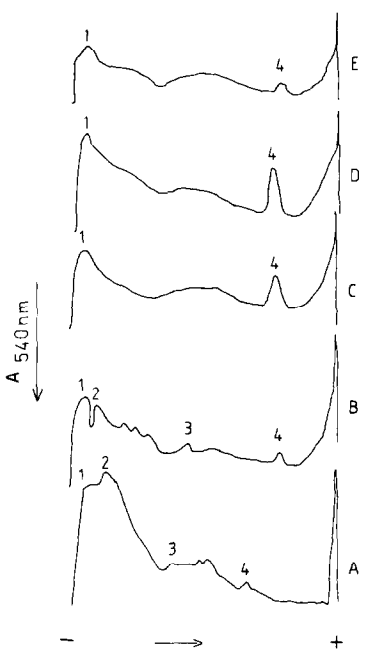

Fig. 5. Densitometric scans of (A) the crude preparation and (B–E) chromatographically purified fractions I–IV, respectively, of *T. verrucosum* proteases, obtained by electrophoresis on polyacrylamide gel containing denatured haemoglobin.

probably forms of proteins with similar electrophoretic mobilities, but with different affinity to the proteins of hen KS, used as a ligand in the chromatographic procedure.

Summarizing the results obtained, it should be emphasized that we have succeeded in developing a simple and cheap method for the chromatographic separation of proteases from *T. verrucosum*, employing for the first time natural keratin protein from hen feathers as a ligand in the affinity procedure. We have also established optimum and repeatable conditions (30 times with good reproducibility) for the separation of proteolytic fractions. This does not mean that proteases from *T. verrucosum* are the only proteases that can be specifically bound by the KS as a ligand. It seems that the proposed procedure may find further applications, especially in biotechnology, in view of its technological and commercial advantages. We have also succeeded in characterizing the proteases contained in the post-culture medium of the fungus *T. verrucosum*. *T. verrucosum* may constitute a good source of these enzymes on a preparative scale, especially when the technique of purification proposed in this paper is used.

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